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# Transfer of antigen between dendritic cells in the stimulation of primary T cell proliferation

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Primary proliferative T cell responses require stimulation with antigen-pulsed dendritic cells (Ag-DC). Here we show that for optimal stimulation, dendritic cells (DC) not exposed directly to antigen are also required. Ag-DC added to DC-depleted T cells caused negligible primary stimulation; adding back DC resulted in stimulation. These effects were seen using the contact sensitizer fluorescein isothiocyanate (FITC), FITC conjugated to ovalbumin (FITC-OVA) or influenza virus as antigens. DC co-cultured with Ag-DC (using FITC or FITC-OVA) acquired antigen indicating that antigen was transferred between DC. DC that acquired antigen secondarily were separated by cell sorting and stimulated primary T cell proliferation directly. DC were also pulsed with FITC, washed thoroughly and incubated overnight. Supernatants contained shed antigen since DC incubated in these supernatants acquired antigen as indicated by flow cytometry. DC acquiring the shed antigen also stimulated T cell proliferation although the stimulation was not as effective as that seen when cell contact between DC and antigen-bearing DC occurred. Thus, in primary stimulation, activation of T cells may occur when there is an antigen gradient between Ag-DC and DC and the mechanisms underlying these effects are now being sought. We propose that this unique interaction between antigen-presenting cells may be a paradigm for self/non-self discrimination.

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## 1 Introduction

The majority of studies *in vitro* have utilized the stimulation of recall responses to antigen added into cultures containing lymphocytes and antigen-presenting cells (APC). Such techniques allow the stimulation of memory T cell proliferation but generally fail to stimulate primary responses in naive T cells. However, the use of potent dendritic cells (DC) expressing antigen can cause the clustering and activation of naive T cells [1–5]. The importance of DC in stimulating high levels of primary T cell responses was confirmed by studies showing that they are also required for effective stimulation of T cells from mice transgenic for T cell receptors with the appropriate stimulating antigen [6, 7]. The reasons for the requirement for antigen-pulsed DC to obtain T cell responses were not clear. However, some authors have described primary stimulation without separating the DC but in these examples, the antigen was also delivered by pulsing an 'antigen-presenting' population with antigen before adding them to responder leukocytes [8, 9]. The presence of one population of cells exposed to antigen added to a responder population not directly exposed to antigen was a common denominator for obtaining primary responses. Partially purified responder T cells were used in some of the experiments where primary stimulation is reported, but none of these studies specifically removed DC from the responder cells. Here we report the unexpected observation that antigen-pulsed DC (Ag-DC) act synergistically with DC not exposed directly to antigen in the production of primary T cell proliferation to antigens.

[I 17839]

**Abbreviation: Ag-DC:** Antigen-pulsed dendritic cells

## 2 Results

### 2.1 Requirement for DC with and DC without antigen

DC pulsed with the antigen FITC were added to nylon wool-enriched T cells from lymph nodes and caused primary T cell proliferation (Fig. 1A). The requirement for

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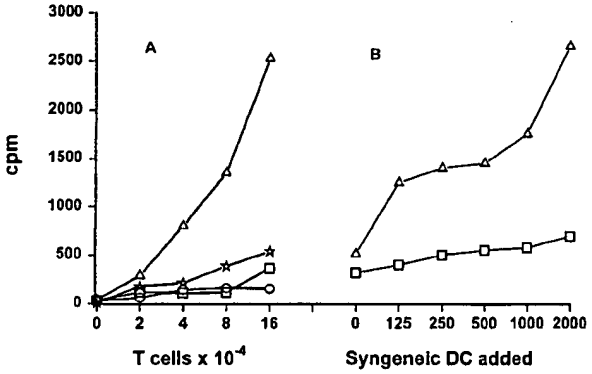


*Figure 1.* Effect of DC without antigen on the stimulation of T cells with FITC-pulsed DC. Enriched T cells treated with complement or enriched T cells depleted of DC with 33D1 and complement were used as responder cells. Proliferative responses to FITC-pulsed DC, measured by [$^3$H]thymidine uptake, in the presence or absence of normal DC are shown in two experiments. (A) DC depletion of responder cells prevented stimulation of responses to FITC-DC. □ Enriched T cells only; ○ Enriched T cells depleted of DC; △ Enriched T cells plus FITC-DC (1000 cells); * Enriched T cells, depleted of DC plus FITC-DC (1000 cells). (B) Addition of as few as 125 normal DC restored responses to FITC-pulsed DC. □ DC-depleted T cells ($16 \times 10^4$) plus 1000 normal DC; △ DC-depleted T cells ($16 \times 10^4$) plus 1000 FITC-pulsed DC. Cultures received in addition 0–2000 normal DC as indicated. Differences 1.9 times background were significant ($p < 0.01$).

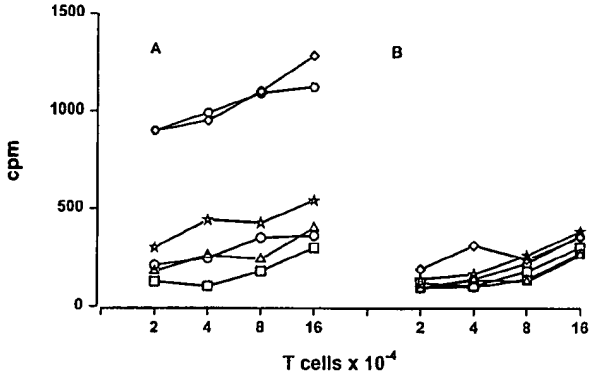


*Figure 2.* DC but not B cells interact to produce T cell responses to FITC. Lymph node T cells depleted of DC were used as responder cells and purified DC and B cells as stimulators. Uptake of [$^3$H]thymidine is shown. (A) DC with antigen interacted with DC without antigen to produce responses. □ DC-depleted T cells only; DC-depleted T cells plus: △ 1000 DC; ○ 1000 FITC-DC; * 2000 FITC-DC; 1000 FITC-DC + 1000 DC; ◇ 2000 FITC-DC + 1000 DC. (B) Using the same responder cells and DC, interaction with B cells failed to stimulate T cell responses to FITC. □ DC-depleted T cells only; DC-depleted T cells plus: △ 2000 B cells; ○ 2000 FITC-B cells; * 2000 FITC-B cells + 2000 B cells; 2000 FITC-DC + 2000 B cells; ◇ 2000 FITC-B cells + 2000 DC. Differences 1.7 times background were significant ($p < 0.01$).

Ag-DC for stimulation of primary FITC responses had previously been established in this system and neither macrophages nor B cells were able to stimulate high responses [3–10]. The DC contaminating the responder T cell population were removed using the specific antibody 33D1 plus complement. The number of cells lost was too small to identify in conventional cell counts. However, cells with the characteristics of DC were no longer seen and no DC could be isolated from these cells using metrizamide gradients. The effectiveness of this treatment was shown by the loss of responsiveness of the T cells to the mitogen Con A (*e.g.* peak responses reduced from 3400 cpm to 340 cpm). Using DC-depleted responder cell populations, addition of DC pulsed with the antigen no longer caused significant levels of primary T cell proliferation (Fig. 1A). Adding back small numbers of DC (*e.g.* 125) caused proliferation of T cells in the presence of the Ag-DC (Fig. 1B). Increasing the number of Ag-DC failed to enhance responses (Fig. 2A). B cells, T cells and macrophages also failed to interact to produce significant proliferation when used as the antigen-pulsed or the non-pulsed APC. A representative experiment of the studies with these other cell types is shown in Fig. 2 where effects of addition of DC and B cells are shown. DC but not B cells exposed to FITC stimulated significant primary proliferative responses when added to T cells but only when DC but not B cells without antigen were also present.

Since FITC is a hapten and binds to many proteins, it was thought that this phenomenon might be specific for haptens. However, similar collaborative effects were seen between DC on stimulation of responses to influenza virus (Fig. 3). Primary responses to FITC conjugated to ovalbumin (FITC-OVA) also showed a similar requirement (not shown).

## 2.2 Transfer of antigen between cells

The possibility of transfer of antigen between DC was tested by mixing DC pulsed with FITC with non-pulsed DC. Fig. 4A and B shows that after 24 h some antigen had been lost from the antigen-pulsed cells and some had been acquired by the normal DC. This effect was not related to the use of a hapten since a similar transfer of antigen was seen using FITC-OVA) (Fig. 4C). The viability of each DC population was similar as indicated from their

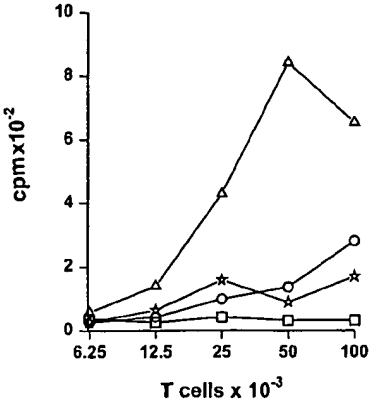


*Figure 3.* DC with and without antigen synergize to produce responses to influenza virus. □ DC-depleted T cells only; DC-depleted T cell plus: O 2000 DC; * 2000 antigen-pulsed DC; △ 1000 antigen-pulsed DC + 1000 DC. Differences 1.8 times background were significant ($p < 0.01$).

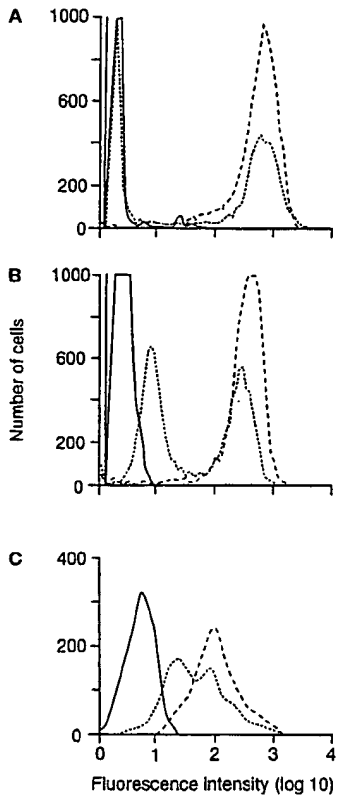


*Figure 4.* Intensity of fluorescence on normal DC, DC exposed to FITC or FITC-OVA *in vitro* and on these populations after overnight co-culture of both DC populations. (A, B) Fluorescence intensity for FITC labeling at 0 h and 18 h at 37 °C, respectively. —— normal DC; --- FITC-DC; ---- Normal DC + FITC-DC. (C) Fluorescence intensity for FITC-OVA labeling at 18 h. —— Normal DC; --- FITC-OVA-DC; ---- Normal DC + FITC-OVA-DC.

characteristic forward and side scatter profiles using the FACScan and from the similar numbers of DC in each population 24 h after mixing (Fig. 4B).

The transfer of antigen was also studied between cell types other than DC (Fig. 5). FITC was used as the antigen in preference to FITC-OVA since the cell populations were discrete by flow cytometry (Fig. 4) and could be sorted. T cells, macrophages, B cells and DC were pulsed with FITC and incubated overnight either on their own or after mixing with equal numbers of non-pulsed cells. In experiments similar to those depicted for DC in Fig. 4A and B, the peak channel of fluorescence for the pulsed (Fig. 5A) and non-pulsed cells (Fig. 5B) in each combination is shown. The DC exposed directly to antigen acquired more antigen than other cells (Fig. 5A). This is demonstrated further for different concentrations of FITC in Table 1 which compares DC and B cells and shows the peak fluorescence after pulsing with different antigen doses. Fig. 5A shows that more antigen was lost from the surface of DC than from other cells when co-cultured with non-pulsed cells overnight. DC also acquired more antigen secondarily from DC than from the other cell types (Fig. 5B). Cells that had acquired antigen from other cells (*i.e.* cells depicted in Fig. 5B) were then separated using the cell sorter and used to stimulate proliferation in DC-depleted autologous T cells. DC that had acquired antigen from Ag-DC caused significant stimulation and marginal effects were seen with B cells that had acquired antigen from DC. This stimulation was not merely related to the level of antigen present on the Ag-DC since Ag-DC pulsed directly with a range of antigen doses failed to cause proliferation in DC-depleted T cells (not shown). In other experiments, DC that had acquired FITC on mixing with FITC-pulsed DC were sorted as described for Fig. 5 and 1000 cells were added to DC-depleted T cells causing direct stimulation as before. Adding back DC without antigen now caused only a marginal increase in stimulation (*e.g.* peak responses of 3250 cpm versus 2910 cpm). DC with antigen acquired indirectly from other DC now stimulated primary proliferation in T cells without the presence of other DC.

## 2.3 Shedding and acquisition of antigen by DC

To find out if the stimulatory antigen was actually shed into the medium by Ag-DC, cells pulsed with FITC or FITC-OVA were washed thoroughly and then incubated at a high cell density overnight ($1 \times 10^6$/0.5 ml medium). Cell-free supernatants of Ag-DC (centrifuged at 12 000 × g) added to syngeneic normal DC resulted in significant acquisition of antigen observed by flow cytometry (Fig. 6). DC exposed to the cell-free supernatants

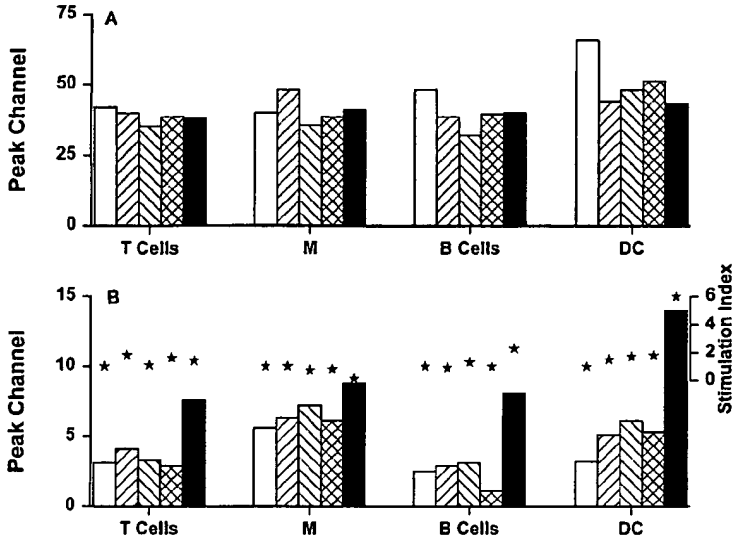


*Figure 5.* Acquisition of FITC directly or indirectly (from other cells) by different cell types, and their stimulatory capacity. (A) Peak fluorescence channel of FITC-pulsed alone □ or co-cultured with T cells ▨, B cells ▩, macrophages ■, or DC ■. (B) Peak fluorescence channel of non-pulsed cells alone □ or co-cultured with FITC-exposed T cells ▩, macrophages ▩, B cells ■, or DC ■. Cells from these peaks were sorted and used to stimulate DC-depleted syngeneic T cells. The stimulation indices for these responses are depicted (*). The results described were obtained in a single large experiment but the findings were confirmed in smaller experiments studying individual cell types.

**Table 1.** Intensity of fluorescence on DC and B cells exposed to varying concentrations of FITC *in vitro*

| Peak fluorescence intensity (log channel no.) | | |
|---|---|---|
| FITC (μg/ml) | DC | B cells |
| 0 | 4.0 | 3.5 |
| 0.125 | 31.5 | 11.8 |
| 1.25 | 43.7 | 23.7 |
| 12.5 | 59.8 | 44.5 |

of Ag-DC also stimulated T cell proliferation. Little stimulation was seen when 2000 DC were used, resulting in stimulation indices of 1.5–2.2, suggesting that synergy between Ag-DC and DC was more efficient with cell contact between DC. However, increasing the number of DC exposed to supernatant to 4000 allowed a peak of response to be obtained (Fig. 7). Supernatants from non-pulsed DC caused little syngeneic T cell stimulation. Stimulatory material in supernatants was unstable since little stimulatory activity was obtained in supernatants left at 37 °C for > 4 h.

Finally, removing FITC from the supernatant with antibody-coated beads resulted in a loss in the stimulatory capacity indicating that the effect was related to the antigen FITC released into the supernatant and not a nonspecific effect of soluble mediators (not shown). Further evidence for this was obtained since cells sorted for the highest level of acquired FITC from the supernatants caused greater stimulation (not shown). Since DC pulsed with FITC were not directly stimulatory and DC exposed to FITC in DC supernatants caused stimulation, alteration of FITC and possibly additional components must be involved.

## 3 Discussion

Antigen present on all DC within a culture failed to activate naive T cells but antigen expressed by only a proportion of DC, where there was an antigen gradient between DC and Ag-DC, caused stimulation of primary responses. This synergy was only relevant for primary stimulation where Ag-DC were required; in secondary responses antigen added directly into cultures containing responder and stimulator cells is stimulatory. The requirement for two signals before DC were active in stimulating primary responses was an unexpected finding, particularly since "purified T cells" have previously

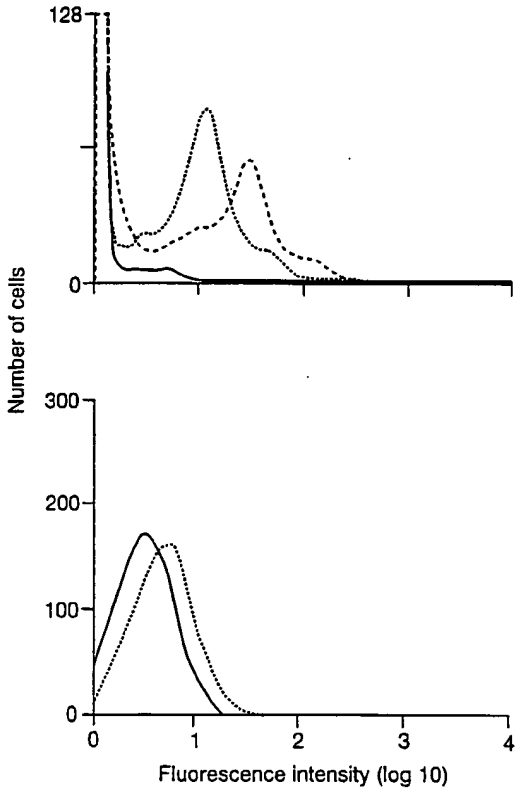


*Figure 6.* Shedding and acquisition of antigen by DC. DC were pulsed for 30 min with antigen and then incubated at $10^6$ cells in 0.5 ml in medium for 24 h. Fresh DC were then separated and "pulsed" with the supernatants of non-pulsed cells or of antigen-pulsed cells. Top: DC were incubated in: — control supernatant; --- supernatant from DC pulsed with 50 μg/ml FITC; ----- supernatant from DC pulsed with 100 μg/ml FITC. Bottom: DC were incubated in: — control supernatant; ----- supernatant from FITC-OVA pulsed DC.

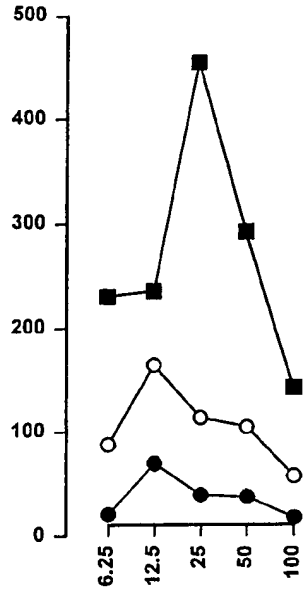


*Figure 7.* Stimulation of T cell proliferation by DC incubated in supernatant of DC exposed to FITC. DC pulsed with 100 μg/ml of FITC or without antigen were washed thoroughly and incubated for 24 h. Fresh DC were incubated for 2 h in the supernatant and 4000 DC added to different numbers of T cells. ● T cells only; O DC exposed to control supernatants; ■ DC exposed to supernatant of antigen-pulsed DC.

been used by ourselves and others as responder cells [2–6]. However, the number of DC required in responder T cells was as low as 1 cell per 2000 lymphocytes. Panning techniques for removal of DC failed to deplete responsiveness. The use of 20-μl hanging drop cultures provides high-density, well-gassed responder cells giving efficient responses to stimulation [5]. In this system, it is possible, even with primary responses, to see not only the linear increase in proliferation at low cell concentrations (as shown in Fig. 1 for primary responses to contact sensitizers) but also, in some instances, the plateau region and area of cell contact inhibition with higher initial cell concentrations (shown for primary responses to influenza virus, Fig. 3). Primary responses to a single peptide often show proliferation only over a narrowly defined range of cell concentrations, although manipulation of the antigen dose and the numbers of stimulator and responder cells could result in wider plateau regions for these responses [3, 5]. In the current studies, a wide plateau region was obtained for responses to contact sensitizers when higher numbers of DC without antigen were added into the cultures and this may reflect the greater efficiency of the stimulation. By contrast, the narrow cell concentration range for the responses and low-level activity seen using DC pulsed with supernatant material may reflect the suboptimal nature of this stimulation. The inefficiency of this latter system is probably reflected in the usual requirement for live metabolically active DC for producing good primary responses *in vitro*.

One major question raised by these results is the identity of the material(s) released by the Ag-DC. The instability of stimulatory materials shed into the supernatants makes direct analysis difficult. There is a precedent for the effectiveness of exosomes in transfer of antigen in work using B cells and cell lines [11]. In attempts to study the possibility that exosomes are shed from DC, supernatants were ultracentrifuged at 150 000 × g. However, because of the inefficiency of the initial supernatant effect and further loss during the control handling

procedures there was little or no activity to study. Some further loss on centrifugation was seen in two experiments (not shown); it is not yet clear whether this indicates that exosomes are involved.

The issue that has been addressed more effectively in the current work is whether the antigen itself is involved. The work shows that Ag-DC can transfer antigen to DC on mixing. It was originally thought that this effect could be specific for contact sensitizers with their propensity to bind to many proteins. However, FITC-OVA was also transferred and Ag-DC plus DC were also required for optimal stimulation with this antigen and with influenza virus. Ongoing experiments are showing that peptide-pulsed DC also release stimulatory molecules into the medium (Iqball and Knight, unpublished). One possibility was that DC may be acquiring cytokines produced by activated DC and causing non-specific activation. However, in experiments where DC were mixed *in vitro* and then sorted, both cell populations were exposed to any nonspecific factors but only the DC which acquired the antigen secondarily were stimulatory. The stimulatory effects of supernatants were also lost when antigen was removed. In addition, DC acquiring antigen from the supernatants of antigen-pulsed DC were sorted according to the level of antigen present; those expressing high antigen levels were more stimulatory than those expressing little. This observation again supports the view that this is an antigen-specific effect although the difference between DC acquiring antigen directly and those acquiring it indirectly means that other factors must be involved. There was a distinction in function between cells that initially acquired antigen and those that presented that antigen to T cells but the basis for this difference is unknown. There may be a requirement for cellular interaction between DC to promote maturation. There are precedents for involvement of cellular interaction for DC maturation; for example, T cell/DC interactions are believed to be required before DC produce IL-12 [12, 13].

High concentrations of DC were required before supernatants were stimulatory and it seems likely that this effect may normally operate by contact between DC. There is a possibility that DC may be heterogeneous or that cells at different stages of maturation may be involved in the shedding and acquisition of antigens, although the flow cytometry experiments suggested that the whole population was involved in antigen transfer. The failure of DC to stimulate responses when an antigen is universally expressed could form the basis for tolerance to shed antigens [14]. It may also be informative to consider neonatal tolerance to high doses of antigen and the involvement of DC in the negative selection of T cells in the light of these studies. By contrast, the stimulation of immunity in neonates by adding DC expressing that antigen may be caused by artificially creating a gradient between DC with and without antigens [15].

We also have evidence that the mixed leucocyte reaction provides a particular example of this phenomenon (Bedford, Garner and Knight, in preparation). DC of the responder type were required for optimal stimulation of responses to allogeneic DC. Responder-type DC pulsed with supernatants of the allogeneic DC stimulated primary mixed leucocyte reaction. This stimulation was blocked by antibodies to the MHC class II of the original allogeneic stimulator cells, demonstrating the specificity of this response. DC also secrete large amounts of MHC molecules (Bedford, Garner and Knight, in preparation) and it may be that the material involves the secretion of MHC plus antigen. A novel mechanism must be proposed since DC do not express antigen receptors of the type found on T cells and involved in specificities of T cell response. However, the internalization of cell surface MHC molecules of DC into vesicles [16–19] may be involved in these effects.

Another question to be considered is the basis for the nonresponsiveness when Ag-DC are added directly to T cells. One possibility is that it is an *in vitro* artefact resulting from the high doses of antigen to which DC may be exposed in direct pulsing experiments. However, the lowest doses of antigen that caused primary stimulation in the mixed cultures were still unable to allow stimulation unless both Ag-DC and DC were present. There was also no evidence that the directly antigen-pulsed cells were damaged either from assessment using dye exclusion tests or from the scatter profile in flow cytometry. If damage to the DC initially pulsed with antigens was involved, the observations would conform to the concept that stimulation occurs when DC detect damaged DC.

The relevance of this observation to immunity *in vivo* is unknown. However, on a few occasions when there was infection within the animal colony, as indicated by enlargement of the lymph nodes or by the pathogen screening, the supernatants of the cells not deliberately pulsed with antigens were stimulatory, masking the antigen-specific effects described in this study. These effects of opportunistic infections suggest that the mechanisms described in this *in vitro* system will have relevance to immune activation *in vivo* but this conclusion remains to be substantiated.

There is one *in vivo* study where a direct effect of addition of DC without antigen was observed, which could be explained on the basis of the interaction between Ag-DC and DC *in vivo*. Mice with a high tumor burden showed no evidence of remission when treated with DC exposed to native tumor antigens, a situation where most DC may

express these antigens. However, complete remission in up to 50 % of the animals was achieved by treating animals with DC without tumor antigens [20]. There is a dichotomy in results in other tumor systems; some benefit by exposure to DC pulsed with a tumor antigen and in other cases anergy may result [21, 22]. It seems possible that antigens which are dominant epitopes and are already expressed on most DC will not be beneficial. On the basis of this hypothesis, injection of "cryptic" or non-dominant antigens either from a tumor or from an organism causing chronic infection may allow expression on some DC and would initiate protective immune responses. There is now some evidence supporting this hypothesis in chronic infection with lymphocytic choriomeningitis virus [23].

A "two cell" hypothesis requiring Ag-DC and DC to produce primary stimulation is compatible with the different effects of antigen dose, receptor frequency and route of immunization. The difficulty in explaining all immunological outcomes on the basis of affinity for TCR may be alleviated if our observation of a requirement for direct evidence of "foreign" or at least different antigens present on a proportion of DC occurs *in vivo*. There is a theory that "danger" is the signal which is involved in initiation of DC maturation to cause primary T cell stimulation [24]. This theory could perhaps be modified to include the concept that detection of "difference" is important in stimulating primary immune responses.

## 4 Materials and methods

### 4.1 Mice

CBA mice between 6 and 12 weeks of age were obtained from the Specific Pathogen Free Unit at Northwick Park Institute for Medical Research. Mice of the same sex were used within experiments.

### 4.2 Cell suspensions

Cell suspensions were prepared from spleens by pressing the tissues through a wire gauze into medium (RPMI 1640, Dutch modification, Flow Laboratories, Irvine, GB) with 100 IU/ml penicillin, 100 µg/ml streptomycin, $5 \times 10^{-5}$ M 2-ME and 10 % FCS. Spleen cells were incubated in flasks (Falcon) overnight and nonadherent cells collected. Nonadherent spleen cells (5–8 ml at around $5 \times 10^6$ cells per ml) were layered onto 2 ml of metrizamide [Nygaard, Oslo, Norway; analytical grade 13.7 % (w/v)], centrifuged for 10 min at 600 × g and the mononuclear cells at the interface collected, washed once and resuspended in medium. These separated cells from specific pathogen-free normal mice were greater than 75 % DC [3], positive for NLDC 145 labeling [25] and sensitive to lysis with a specific antibody for DC (33D1) plus complement [26]. Less than 3 % were macrophages which could be labeled with the F4/80 mAb [27] and the remaining cells were T lymphocytes. Peritoneal exudate macrophages were separated from animals 3 days after i.p. injection of 60 µg Con A as a source of cells expressing MHC class II molecules [10]. DC were removed by treatment with 33D1 and complement and the remaining cells were > 90 % F4/80-positive macrophages.

Pooled inguinal, axillary, brachial and popliteal lymph nodes from normal CBA mice were used as a source of responder T cells. Enriched T cells (greater than 85 %) were obained by passage of lymph node cells over nylon wool columns [28]. Enriched B cells (80 %) were obtained by disruption of the nylon wool to elute loosely adherent cells and these were incubated for a further 1 h on plastic to remove macrophages and treated with anti-Thy 1.2 and 33D1 plus complement to remove T cells and DC.

### 4.3 Flow cytometry

Fluorescence intensity was measured and in some experiments highly purified cells were obtained by fluorescence-activated cell sorting from the partially enriched populations using the FACSTAR plus (Becton Dickinson & Co., Mountain View, CA). Propidium iodide staining was used to identify dead cells. Cells were identified using specific mAb already described and the appropriate regions were gated to ensure purification of the specific cell populations. For FCM analysis, $2 \times 10^5$ cells were usually counted in each sample and the FACSTAR was set up with the laser set at 300 mW, 488 mm and photomultiplier tube at 550 V with fluorescence intensity displayed using a logarithmic amplifier. In some experiments the FACScan was used for analysis of expression of FITC where sorting was not required.

### 4.4 Antigen pulsing

Between $1 \times 10^5$ and $1 \times 10^6$ of the DC-enriched population separated from the spleen cells or other cell populations were added to 1 ml solutions containing different concentrations of FITC (Isomer 1, Sigma, GB). The cell suspension was incubated at 37 °C for 20 min and then washed three times with cold medium. Some DC were sensitized by pulsing with X47 influenza virus [3] or with FITC-OVA [21] for 2 h and washed before use.

### 4.5 Antibody depletion

Lysis with antibody plus complement was used to deplete cell preparations of DC using 33D1 [25] or of T cells using anti-thy 1.2 (AT83a). Cells at $5 \times 10^5$–$5 \times 10^6$/ml were mixed with antibody and rabbit serum as a source of complement (Buxted Rabbit Co., Buxted, GB) or with complement alone

as a control for cytotoxicity, incubated for 60 min at 37 °C and washed twice in medium. No DC could be identified in the cells depleted by this method which was used in preference to panning or magnetic bead techniques which may be less efficient due to the clustering of DC with other cell types.

### 4.6 *In vitro* stimulation assay

Enriched T cells or enriched T cells with DC removed by antibody and complement treatment were cultured in triplicate at different concentrations of viable (dye-excluding) cells in 20-μl hanging drop cultures in Terasaki plates and stimulated by DC or other cell populations, some of which were pulsed with antigens. After 3 days the cultures received 1 μl [$^3$H]thymidine (Amersham International, Amersham, GB) at a specific activity of 2 Ci/mM, giving a final concentration of 1 μg/ml of thymidine. After 2 h the cultures were blotted onto filter discs and washed with saline, trichloroacetic acid and methanol and counted in a scintillation counter. These conditions for labeling resulted in "flooding" conditions throughout the pulse time and low radiation damage. They gave low counts but these accurately reflect the DNA synthesis [29]. This system was previously shown to allow the development of primary proliferative and cytotoxic T cell responses *in vitro* to protein and peptide antigens and to FITC [3, 5, 10]. In this system maximal responses to the mitogen Con A were between 2000 and 5000 cpm and primary responses to antigens between 300 and 3500 cpm. Variability in triplicates was generally within 20 % of the mean and analysis of variance of log transformed data to assess significance of differences in cpm showed that a doubling of counts indicated $p < 0.01$.

### 4.6 Removal of antigen from DC supernatants

Magnetic beads with anti-FITC or an isotype control antibody were added to the supernatants of some FITC-pulsed DC after overnight culture. The beads were then removed using MINIMACS columns (Miltenyi Biotech, Bisley, GB) and the depleted supernatants used in stimulation assays.

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